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5807300602

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5. Name of your agent *(if you have one)* — Carol P Greaves

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12. Name and daytime telephone number of person to contact in the United Kingdom Dr S Kremer 0117 91 32861

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PESTICIDAL AGENTS

The present invention relates to materials, agents and compositions having pesticidal activity which derive from bacteria, and more particularly from Xenorhabdus species. The invention further relates to organisms and methods employing such compounds and compositions.

There is an ongoing requirement for materials, agents, compositions and organisms having pesticidal activity, for instance for use in crop protection or insect-mediated disease control. Novel materials are required to overcome the problem of resistence to existing pesticides. Ideally such materials are cheap to produce, stable, have a high toxicity (either when used alone or in combination) and are effective when taken per os by the pest target. Thus any invention which provided materials, agents, compositions or organisms in which any of these properties was enhanced would represent a step forward in the art.

Xenorhabdus spp. in nature are frequently symbiotically associated with a nematode host, and it is known that this association may be used to control pest activity. For instance, it is known that certain Xenorhabdus spp. alone are capable of killing an insect host when injected into the host's hemocoel.

In addition, one extracellular insecticidal toxin from Photorhabdus luminescens has been isolated (this species was recently removed from the genus Xenorhabdus, and is closely related to the species therein). This toxin is not effective when ingested, but is highly toxic when injected into certain insect larvae (see Parasites and Pathogens of Insects Vol.2, Eds. Beckage, N. E. et al., Academic Press 1993).

Also known are certain low-molecular weight heterocyclic compounds from P. luminescens and X. nematophilus which have antibiotic properties when applied intravenously or topically (see Rhodes, S.H. et al., PCT WO 84/01775).

Unfortunately none of these prior art materials have the ideal pesticide characteristics discussed above, and in particular, they do not have toxic activity when administered orally.

The present invention provides pesticidal agents and compositions from Xenorhabdus species, organisms which produce such compounds and compositions, and methods which employ these agents, compositions and organisms, that alleviate some of the problems with the prior art.

According to a first aspect of the present invention there is disclosed a method of killing pests comprising administering cells from Xenorhabdus species or pesticidal materials derived therefrom orally to the pests.

A PCT application of CSIRO published as WO 95/00647 discloses an apparently toxic protein from Xenorhabdus nematophilus; however no details of the protein's toxicity are given, and certainly there is no disclosure of its use as an oral insecticide.

The term 'Xenorhabdus species' is intended to embrace also those sepcies which are closely related to Xenorhabdus, or have recently been removed from that genus (such as P. luminescens). Preferably the bacteria are X. nematophilus. Preferably the pest target is an insect, and more preferably it is of the order Lepidoptera, particularly Pieris brassicae, Pieris rapae, or Plutella xylostella or the order Diptera, particularly Culex quinquefaciatus.

In preferred forms of the invention, cells from Xenorhabdus species are used in conjunction with Bacillus thuringiensis as an oral pesticide.

In further embodiments, rather than using Bacillus thuringiensis itself, pesticidal materials derived from B. thuringiensis (eg. delta endotoxins or other isolates) are used in conjunction with Xenorhabdus species.

The term 'derived from' is intended to embrace not only materials which have been isolated directly from the bacterium in question, but also those which have been subsequently cloned into and produced by other organisms.

Thus the unexpected discovery that bacteria of the Xenorhabdus species (and materials derived therefrom) have pesticidal activity when ingested, and that such bacteria and materials can be used advantageously in conjunction with B. thuringiensis (and toxins or materials derived therefrom), forms the basis of the present invention. The pesticidal activity of B. thuringiensis isolates alone have been well documented. However, synergistic pesticidal activity between such isolates and bacteria of the Xenorhabdus species (or materials derived therefrom) has not previously been demonstrated.

In still further embodiments of the invention culture supernatant taken from cultures of Xenorhabdus species, particularly X. nematophilus, is used in place of cells from Xenorhabdus species in the methods above.

All of these methods can be employed, *inter alia*, in pest control.

The invention also makes available pesticidal compositions comprising cells from Xenorhabdus species, preferably X. nematophilus, in combination with B. thuringiensis.

As with the methods above, a pesticidal toxin from B. thuringiensis (preferably a delta endotoxin) may be used as an alternative to B. thuringiensis in the compositions of the present invention. Likewise, culture supernatant taken from cultures of Xenorhabdus species, preferably, X. nematophilus may be used in place of cells from Xenorhabdus species.

Such compositions can be employed, *inter alia*, for crop protection eg. by spraying crops.

The invention also discloses an isolated pesticidal agent characterised in that the agent is obtainable from cultures of X. nematophilus or mutants thereof, has oral pesticidal activity against Pieris brassicae, Pieris rapae and Plutella xylostella, is substantially heat stable to 55°C, is proteinaceous, acts synergistically with B. thuringiensis cells as an oral pesticide, is substantially resistant to proteolysis by trypsin and proteinase K and has a molecular weight of between 30 and around 100 kDa.

By 'substantially heat stable to 55°C' is meant that the agent retains some pesticidal activity when tested after heating the agent in suspension to 55°C for 10 minutes, and preferably retains at least 50% of the untreated activity.

By 'substantially resistant to proteolysis' is meant that the agent retains some pesticidal activity when exposed to the proteases at 30°C for 2 hours and preferably retains at least 50% of the untreated activity.

By 'acts synergistically with B. thuringiensis cells as an oral pesticide' is meant that the concentration of B. thuringiensis cellular material necessary to give 50% mortality in a P. brassicae when used alone is reduced by at least 80% when it is used in combination the agent at a concentration sufficient to give 25% mortality when the agent is used alone.

The tentative assignment of a molecular weight between 30 and 100 kDa has been made on the basis that the activity is retained by 30 kDa cut-off filters but is only partly retained by 100 kDa filters.

Preferably the agent is still further characterised in that the pesticidal activity is lost through treatment at 25°C with sodium dodecyl sulphate (SDS - 0.1% 60 mins) or Acetone (50% 60 mins).

Clearly the characterising properties of the isolated agent described above can be utilised to purify it from, or enrich its concentration in, Xenorhabdus species cells and culture medium supernatants.

Methods of purifying proteins from heterogenous mixtures are well known in the art (eg. ammonium sulphate precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration, etc.). The oral pesticidal activity provides a convenient method of assaying the level of agent after each stage, or in each sample of eluent. Such methodology does not require inventive endeavour by those skilled in the art.

The invention further discloses oral pesticidal compositions comprising one or more agents as described above. Such compositions preferably further comprise other pesticidal materials from none-Xenorhabdus species. These other materials may be chosen such as to have complementary properties to the agents described above, or act synergistically with it.

Preferably the oral pesticidal composition comprises one or more pesticidal agents as described above in combination with B. thuringiensis (or with a toxin derived therefrom, preferably delta endotoxin).

The invention further makes available a host organism comprising a nucleotide sequence coding for an agent as described above.

Methods of cloning the sequence for a characterised protein into a host organism are well known in the art. For instance the protein may be purified and sequenced: as activity is not required for sequencing, SDS gel electrophoresis followed by blotting of the gel may be used to purify the protein. The protein sequence can be used to generate a nucleotide probe which can itself be used to identify suitable genomic fragments from a Xenorhabdus gene library. These fragments can then be inserted via a suitable vector into a host organism which can express the protein. The use of such general methodology is routine and non-inventive to those skilled in the art.

It may be desirable to manipulate (eg. mutate) the agent by altering its gene sequence (and hence protein structure) such as to optimise

its physical or toxicological properties.

It may also be desirable for the host to be engineered or selected such that it also expresses other proteinaceous pesticidal materials (eg. delta- endotoxin from B. thuringiensis). Equally it may be desirable to generate host organisms which express fusion proteins composed of the active portion of the agent plus these other toxicity enhancing materials.

A host may be selected for the purposes of generating large quantities of pesticidal materials for purification eg. by using B. thuringiensis transformed with the agent-coding gene. Preferably however the host is a plant, which would thereby gain improved pest-resistance. Suitable plant vectors, eg. the Ti plasmid from Agrobacterium tumefaciens, are well known in the art. Alternatively the host may be selected such as to be directly pathogenic to pests, eg. an insect baculovirus.

The teaching and scope of the present invention embraces all of these host organisms plus the agents, mutated agents or agent-fusion materials which they express.

Thus the invention makes available methods, compositions, agents and organisms having industrially applicable pesticidal activity, being particularly suited to improved crop protection or insect-mediated disease control.

The methods, compositions and agents of the present invention will now be described, by way of illustration only, through reference to the following non-limiting examples and figures. Other embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

FIGURE

Figure 1 shows the variation with time of the growth of X.

nematophilus 9965 and activity of cells and supernatants against P. brassicae as described in Example 3.

EXAMPLES

Example 1 - Use of X. nematophilus cells as oral insecticide

CELL GROWTH: A subculture of X. nematophilus (ATCC 19061, Strain 9965 available from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland) was used to inoculate 250 ml Erlenmeyer flasks each containing 50 ml of Luria Broth containing 10g tryptone, 5g yeast extract and 5g NaCl per litre. Cultures were grown in the flasks at 27°C for 40 hrs on a rotary shaker.

PRODUCTION OF CELL SUSPENSION: Cultures were centrifuged at 5000g for 10 mins. The supernatants were discarded and the cell pellets were washed once and resuspended in an equal volume of phosphate buffered saline (8g NaCl, 1.44g Na_2HPO_4 and 0.24g of KH_2PO_4 per litre) at pH 7.4.

ACTIVITY OF CELL SUSPENSION TO INSECTS: The Bioassays were as follows: P. brassicae: The larvae were allowed to feed on an artificial agar-based diet (as described by David and Gardiner (1965) London Nature, 207, 882-883) into which a series of dilutions of cell suspension had been incorporated. The bioassays were performed using a series of 5 doses with a minimum of 25 larvae per dose. Untreated and heat-treated (55°C for 10 minutes) cells were tested. Mortality was recorded after 2 and 4 days with the temperature maintained at 25°C.

	LC_{50} cells/g diet	
Treatment	2 days	4 days
Untreated	5.9×10^5	9.8×10^4
Treated 55°C	7.1×10^5	1.4×10^5

Aedes aegypti: The larva were exposed to a series of 5 different

dilutions of cell suspension in deionised water. The biosassays were performed using 2 doses per dilution of 50 ml cell suspension in 9.5 cm plastic cups with 25 second instar larvae per dose. Untreated and heat-treated (55°C or 80°C for 10 minutes) cells were tested. Mortality was recorded after 2 days with the temperature maintained at 25°C.

Treatment	LC_{50} cells/ml 2 days
Untreated	5.1×10^6
Treated 55°C	7.4×10^6
Treated 80°C	$> 10^8$

Culux quinquefaciatus: The larva were exposed to a single concentration cell suspension containing 4×10^7 cells/ml. The biosassays were performed using 2 doses of 50 ml cell suspension in 9.5 cm plastic cups with 25 second instar larvae per dose. Untreated and heat-treated (55°C or 80°C for 10 minutes) cells were tested. Mortality was recorded after 2 days with the temperature maintained at 25°C.

Treatment	% Mortality 2 days
Untreated	100
Treated 55°C	100
Treated 80°C	0

Thus these results clearly show that cells from X. nematophilus are effective as an oral insecticide against a number of insect species (and are particularly potent against P. brassicae). The insecticidal activity is not dependent on cell viability (i.e is largely unaffected by heating to 55°C which reduces cell viability by >99.99%) but is much reduced by heating to 80°C, which denatures most proteins.

Example 2 - Use of X. nematophilus supernatant as an oral insecticide

CELL GROWTH: Cultures were grown as in Example 1.

PRODUCTION OF SUPERNATANT: Cultures were centrifuged twice at 10000g for 10 mins. The cell pellets were discarded.

ACTIVITY OF SUPERNATANT TO INSECTS: The Bioassay was as follows: Activity against neonate P. brassicae and two day old Pieris rapae and Plutella xylostella larvae was measured as for P. brassicae in Example 1, but using a series of untreated dilutions of supernatant in place of of cell supensions and with mortality being recorded after 4 days only.

	LC_{50} µl supernatant/g diet
Insect species	4 days
P. brassicae	22
P. rapae	79
P. xylostella	135

In addition, size-reducing activity (62% reduction in 7 days) against Mamestra brassicae was detected in larvae fed on an artificial diet containing X. nematophilus supernatant (results not shown).

Thus these results clearly show that the supernatant from X. nematophilus culture medium is effective as an oral insecticide against a number of insect species, and are particularly potent against P. brassicae.

The heating of supernatants to 55°C for 10 minutes caused a partial loss of activity while 80°C caused complete loss of activity. Activity was also completely lost by treatment with SDS (0.1% 60 mins) and Acetone (50% 60 mins) but was unaffected by Triton X-100 (0.1% 60 mins), non-diet P40 (0.1% 60 mins), NaCl (1 M 60 mins) or cold storage at 4°C or -20°C for 2 weeks. All of these properties are consistent with a proteinaceous agent.

The general mode of action of X. nematophilus cells and supernatants

i.e. reduction in larval size and death within 2 days at high dosages, and other properties, eg. temperature resistence, appear to be similar suggesting a single agent or type of agent may be responsible for the oral insecticide activity activities of both cells and supernatants.

Example 3 - Timescale for appearance of ingestable insecticide activity

CELL GROWTH: 1ml of an overnight culture of X. nematophilus was used to inoculate an Erlenmeyer flask. Cells were then cultured as in Example 1. Growth was estimated by measuring the optical density at 600 nm.

PRODUCTION OF CELL SUSPENSION AND SUPERNATANTS: These were produced as in Examples 1 and 2.

ACTIVITY OF CELLS AND SUPERNATANTS AGAINST P. BRASSICAE: The cell suspension bioassay was carried out as in Example 1, but using a single dose of suspended cells equivalent to 50 µl of broth/g diet and measuring mortality after 2 days. The cell supernatant bioassay was carried out as in Example 2, but using a single dose equivalent to 50 µl supernatant/g diet (i.e. more than twice the LC_{50}) and measuring mortality after 2 days.

The results are shown in Fig. 1. Thus these results clearly show that cells taken from X. nematophilus culture medium are highly effective as an oral insecticide against P. brassicae after only 5 hours, and supernatants are highly effective after 20 hours. Although some slight cell lysis was observed in the early stages of growth, no significant cell lysis was observed after this point demonstrating that the supernatant activity may be due to an authentic extracellular agent (as opposed to one released only after cell breakdown).

Example 4 - Synergy between of X. nematophilus cells and B. thuringiensis powder preparations

CELL GROWTH AND SUSPENSION: X. nematophilus cells were grown and suspended as in Example 1. B. thuringiensis strain HD1 (from Bacillus Genetic Stock Centre, The Ohio State University, Columbus, Ohio 43210, USA) was cultured, harvested and formulated into a powder as described by Dulmage et al.(1970) J. Invertebrate Pathology 15, 15-20.

ACTIVITY OF X. NEMATOPHILUS CELLS AND B. THURINGIENSIS POWDER AGAINST P. BRASSICAE: The Bioassays was carried out using X. nematophilus and B. thuringiensis in combination or using B. thuringiensis cell powder alone. Bioassays were carried out as in Example 1 but with various dilutions of B. thuringiensis powder in place of X. nematophilus. For the combination experiment, a constant dose of X. nematophilus cell suspension sufficient to give 25% mortaility was also added to the diet. Mortality was recorded after 2 days.

	LC_{50} µg Bt powder/g diet
Bioassay	2 days
B.t. alone	1.7
B.t. plus X.n.	0.09

These results clearly demonstrate the synergism between X. nematophilus cells and B. thuringiensis powder when acting as an oral insecticide against P. brassicae.

Example 5 - Synergy between of X. nematophilus supernatants and B. thuringiensis powder

CELL GROWTH AND PRODUCTION OF SUPERNATANTS: X. nematophilus cells were grown and supernatants prepared as in Example 2. B. thuringiensis was grown and treated as in Example 4.

ACTIVITY OF Xn SUPERNATANTS AND Bt CELL POWDER AGAINST P. BRASSICAE: The Bioassays were carried out using X. nematophilus supernatants and B. thuringiensis in combination or using B. thuringiensis powder alone. The Bioassay against neonate P. brassicae and two day old Pieris rapae and Plutella xylostella larvae were measured as in Example 2 but with various dilutions of B. thuringiensis in place of X. nematophilus. For the combination experiment, a constant dose of X. nematophilus supernatant sufficient to give 25% mortality was also added to the diet. Mortality was recorded after 4 days.

	LC_{50} µg Bt powder/g diet	
Insect species	Bt alone	Bt plus Xn
P. brassicae	1.4	0.12
P. rapae	2.5	0.26
P. xylostella	7.2	0.63

These results clearly demonstrate the synergism between X. nematophilus supernatants and B. thuringiensis powder when acting as an oral insecticide against several insect species. The fact that both X. nematophilus cells and supernatants demonstrate this synergism strongly suggests that a single agent or type of agent is responsible for the demonstrated activities.

Example 5 - Characterisation of insecticidal agent from X. nematophilus supernatant by proteolysis

CELL GROWTH AND PRODUCTION OF SUPERNATANTS: X. nematophilus cells were grown and supernatants prepared as in Example 2.

PROTEOLYSIS OF SUPERNATANT: Culture supernatant (50ml) was dialysed against 0.5 M NaCl (3 x 1 l) for 48 hours at 4°C. The volume of the supernatant in the dialysis tube was reduced five-fold by covering with polyethylene glycol 8000 (Sigma chemicals). Samples were removed and treated with either trypsin (Sigma T8253 = 10,000 units/mg) or proteinase K (Sigma P0390 = 10 units/mg) at a concentration of 0.1 mg protease/ml sample for 2 hours.

ACTIVITY OF PROTEASE TREATED SUPERNATANT AGAINST P. BRASSICAE: The Bioassay against neonate P. brassicae larvae was carried out by spreading 25 µl of each 'treatment' on the artificial agar-based diet referred to in Example 1 in a 4.5 cm diameter plastic pot. Four pots each containing 10 larvae were used for each treatment. Mortalities were recorded after 1 and 2 days. Controls using water only, trypsin (0.1 mg/ml) and proteinase K (0.1 mg/ml) were also tested in the same way.

	% Mortality	
Treatment	1 day	2 days
Untreated supernatant	60	100
Proteinase K treated supernatant	45	100
Trypsin treated supernatant	40	100
All controls (no supernatant)	0	0

CLAIMS

1. A method for killing pests comprising administering cells from Xenorhabdus species or pesticidal materials derived therefrom orally to the pests.

2. A method as claimed in claim 1 wherein the cells from Xenorhabdus species or the pesticidal materials derived from such cells are administered in conjunction with Bacillus thuringiensis or pesticidal materials derived therefrom.

3. An pesticidal composition comprising cells from Xenorhabdus species or the pesticidal materials derived from such cells in combination with B. thuringiensis or pesticidal materials derived therefrom.

4. A method or composition as claimed in any one of the preceding claims wherein the Xenorhabdus species is X. nematophilus.

5. A method or composition as claimed in any one of the preceding claims wherein the pesticidal materials derived from cells from Xenorhabdus species are derived from supernatant taken from cultures of such cells.

6. A method or composition as claimed in any one of the preceding claims wherein the pesticidal materials derived from B. thuringiensis comprise the delta endotoxin.

7. A method or composition as claimed in any one of the preceding claims wherein the pests are insects.

8. A method or composition as claimed in claim 7 wherein the insects are from the order Lepidoptera or Diptera.

9. An isolated pesticidal agent characterised in that it is obtainable from cultures of X. nematophilus or mutants thereof, has oral pesticidal activity against Pieris brassicae, Pieris rapae and

Plutella xylostella), is substantially heat stable to 55°C, is proteinaceous, acts synergistically with B. thuringiensis cells as an oral pesticide, is substantially resistant to proteolysis by trypsin and proteinase K and has a molecular weight of between 30 and around 100 kDa.

10. An isolated pesticidal agent as claimed in claim 9 further characterised in that the pesticidal activity is substantially destroyed by treatment with sodium dodecyl sulphate or acetone or heating to 80°C.

11. An isolated pesticidal agent as claimed in claim 9 or claim 10 further characterised in that the the agent is an extracellular protein.

12. An isolated pesticidal agent as claimed in any one of claims 9 to 11 further characterised in that the the agent is a protein having a molecular weight of around 45 kDa.

13. An pesticidal composition comprising one or more agents as claimed in any one of claims 9 to 12.

14. A composition as claimed in claim 13 further comprising B. thuringiensis or pesticidal materials derived therefrom.

15. A composition as claimed in claim 14 wherein the pesticidal materials derived from B. thuringiensis comprise the delta endotoxin.

16. A host organism comprising a nucleotide sequence coding for an agent as claimed in any one of claims 9 to 12.

17. A host organism comprising a nucleotide sequence which is at least 90% homologous with a nucleotide sequence coding for an agent as claimed in any one of claims 9 to 12.

18. A host organism as claimed in claim 16 or 17 which has been

engineered or selected such that it also expresses other pesticidal proteinaceous toxicity enhancing materials

19. A host organism comprising a nucleotide sequence coding for a fusion protein comprising the active portion of an agent as claimed in any one of claims 9 to 12 in combination with other pesticidal proteinaceous toxicity enhancing materials.

20. A host organism as claimed in claim 16 or 17 wherein the pesticidal toxicity enhancing materials comprise delta- endotoxin from B. thuringiensis).

21. A host organism as claimed in any one of claims 16 to 20 wherein the host is a plant.

22. A host organism as claimed in any one of claims 16 to 20 wherein the host is a virus pathogenic to insects.

23. A fusion protein as expressed by a host as claimed in claim 19.

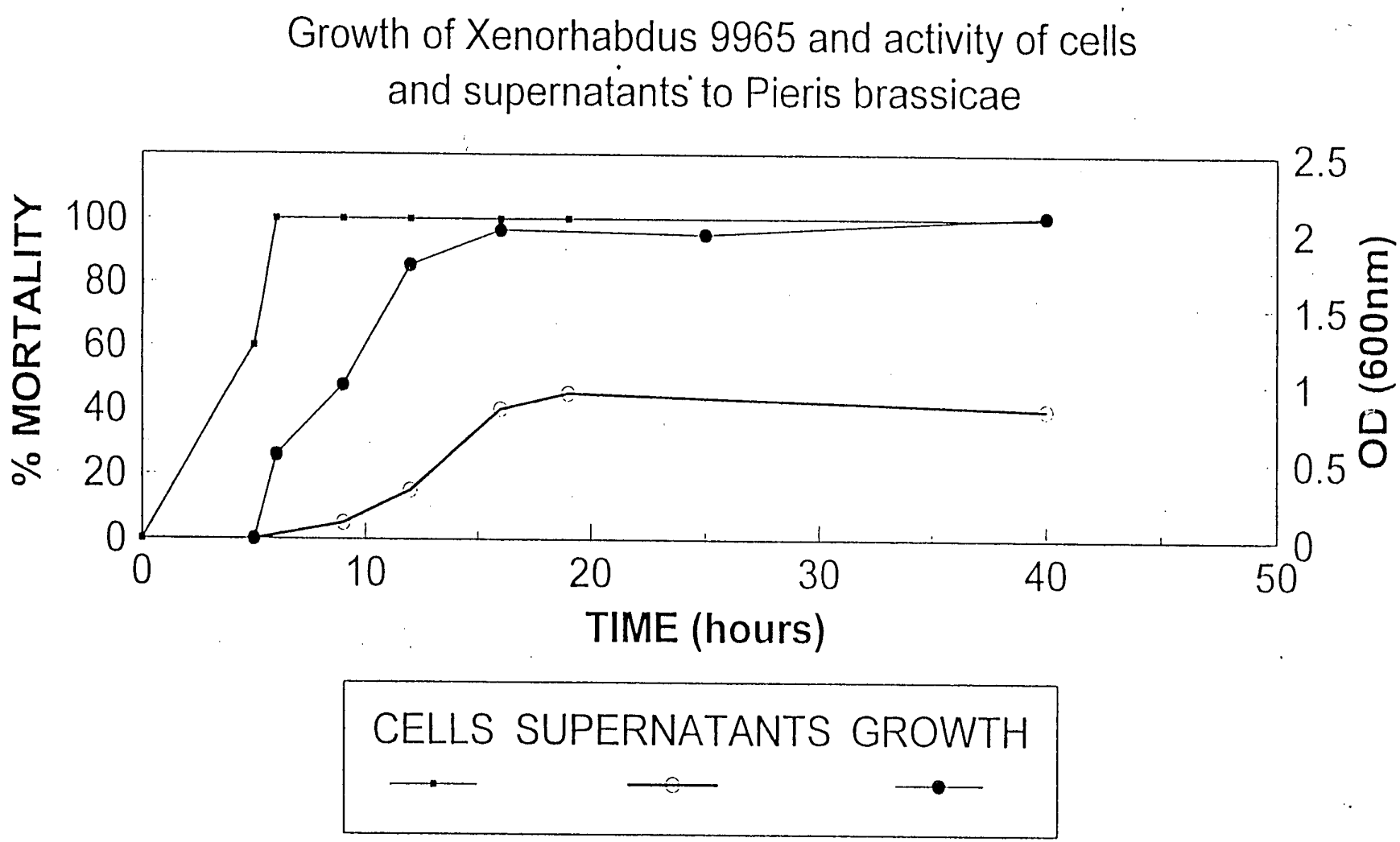
24. An pesticidal agent substantially as described hereinbefore with reference to Figures 1 to 3 and Examples 1 to 6.

ABSTRACT

PESTICIDAL AGENTS

A method for killing pests (eg. insects) comprising administering cells from Xenorhabdus species (eg. X. nematophilus) or pesticidal materials derived therefrom orally to the pests, either alone or in conjunction with Bacillus thuringiensis or pesticidal materials derived therefrom. Also disclosed is an isolated pesticidal agent (and compositions comprising the same) characterised in that it is obtainable from cultures of X. nematophilus or mutants thereof, has oral pesticidal activity against Pieris brassicae, Pieris rapae and Plutella xylostella), is substantially heat stable to 55°C, is proteinaceous, acts synergistically with B. thuringiensis cells as an oral pesticide, is substantially resistant to proteolysis by trypsin and proteinase K and has a molecular weight of between 30 and around 100 kDa.

Figure 1.


Growth of Xenorhabdus 9965 and activity of cells and supernatants to Pieris brassicae
% MORTALITY
100
80
60
40
20
0
0
10
20
30
40
50
TIME (hours)
2.5
2
1.5
1
0.5
0
OD (600nm)
CELLS
SUPERNATANTS
GROWTH

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29-8-97

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